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# Stem Cell Delivery with Polymer Hydrogel for Treatment of Intervertebral Disc Degeneration: From 3D Culture to Design of the Delivery Device for Minimally Invasive Therapy

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**Running head:** Amniotic membrane attenuates biliary fibrosis

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**ABSTRACT**

Nucleus pulposus (NP) tissue damage can induce detrimental mechanical strain on the biomechanical performance of intervertebral disc (IVD) causing subsequent disc degeneration. A novel, photocurable, injectable, synthetic polymer hydrogel (pHEMA-co-APMA grafted with PAA) has already demonstrated success in encapsulating and differentiating human mesenchymal stem cells (hMSCs) towards an NP phenotype during hypoxic conditions. After demonstration of promising results in our previous work, in this study, we have further investigated the inclusion of mechanical stimulation and its impact on hMSC differentiation towards an NP phenotype through the characterization of matrix markers such as SOX-9, Aggrecan and Collagen II. Furthermore, investigations were undertaken in order to approximate delivery parameters for an injection delivery device, which could be used to transport hMSCs suspended in hydrogel into the IVD. hMSC laden hydrogel solutions were injected through various needle gauge sizes in order to deter its impact on post-injection cell viability and IVD tissue penetration. Interpretation of this data informed the design of a potential minimally invasive injection device, which could successfully inject hMSCs encapsulated in a UV curable polymer into NP, prior to photocrosslinking in situ.

**Keywords:** regenerative medicine, cell encapsulation, tissue engineering, cell delivery, device design

## INTRODUCTION

Biomechanical and pathohistological structure changes occur in the degenerated intervertebral disc (IVD). The difficulty in IVD tissue to repair and regenerate itself is attributed to its relatively acellular (5000 cells/mm$^3$), acidic and avascular environment. Subsequently, when considering an acellular or tissue engineering therapy for the IVD, several factors must be considered including: extracellular matrix architecture, hydrated tissue matrix, hypoxic nature and the mechanical stresses and strains imposed upon the nucleus pulposus (NP) tissue. For this reason, hydrogels are an ideal and popular choice, as they are able to offer a hydrated environment, the ability to mimic native fibrillar structure and are biocompatible [1,2]. Furthermore, photocurable hydrogels display additional benefits, including fine-tuning mechanical properties and swelling behavior, short-reaction times, minimal heating and minimally invasive delivery. To date, only a single study exists which has displayed the successful use of a photocurable polyethyelene glycol diacrylate hydrogel to encapsulate and differentiate MSCs towards NP-like cells, proven by the positive expression of typical NP matrix proteins (Aggrecan and Collagen II) [3].

Various parameters have been demonstrated to influence the differentiation of hMSCs towards an NP cell-like phenotype including: a 3D environment supported by hydrogels [4], hypoxia and chemical stimulation and disc microenvironment [5]. A study by Stoyanov et al demonstrated the successful differentiation of hMSCs towards NP cells under the combined influence of 3D encapsulation using alginate beads and chemical media [6]. Recently, our group demonstrated the successful use of a novel, synthetic, UV photocurable hydrogel p(HEMA-co-APMA)g PAA for the encapsulation of hMSCs and their differentiation towards an NP- cell like phenotype [7]. In this case, differentiation was achieved through the multiple combinatorial effects



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of 3D encapsulation, chondrogenic media and hypoxia. In this study, we have further investigated the added influence of mechanical stimulation to hMSC differentiation and also demonstrated the feasibility of cell delivery using injection devices.

## MATERIALS AND METHODS

### *Preparation of Photocurable Hydrogel Solution*

pHEMA-APMA-PAA polymer was prepared as described previously [7]. Briefly, hydrogels were prepared at 13.5 % (w/v) concentration by dissolving the required amount of polymer in phosphate buffer saline (PBS). 50 % (w/v) Irgacure 2959 (prepared using DMSO) solution was added to the polymer solution at 1 % volume of the total polymer solution. Polymer solution was briefly vortexed and sterile filtered before use.



### *hMSC, expansion, Encapsulation in Hydrogels and Differentiation towards NP like Cells*

hMSCs from fresh human bone marrow aspirate (Lonza, UK) was recovered, isolated and expanded as described previously by Pittenger *et. al.* [8]. hMSCs were expanded using hMSC expansion media which consisted of: DMEM, 10% Fetal bovine serum (FBS; Lonza, UK), 1% L-glutamine (L-glut; Lonza, UK), 1% non-essential amino acids (NEAA; Lonza, UK) and 1% penicillin and streptomycin and incubated in an hypoxic chamber at 2% $O_2$ SCI-Tive workstation (RUSKINN Technology, The Baker Company). Seeding solutions consisted of 200 000 cells/50 µl of hMSC media per hydrogel. Cell seeding solution and cross-linking was performed as mentioned in our previous work [7]. Chondrogenic media (consisting of: DMEM, 1% FBS, 1% NEAA, 1% L-glut, 0.1µM dexamethasone, 50µM ascorbic acid, 40µg $ml^{-1}$ L-proline, 1%



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sodium pyruvate and 10ng $ml^{-1}$ TGF-β3) was conditioned in hypoxia at 2% $O_2$ using the SCI-Tive workstation for 24 hours, prior to use. Mechanical stimulation to biological samples was applied using the BOSE bioreactor. Stimulation parameters of cyclic compression (5%) at 1Hz for 15 mins a day, up to day 7 or 14 were applied. Hydrogels encapsulated with hMSCs cultured under hMSC or chondrogenic media, under hypoxia were loaded into the biodynamic chamber using sterile forceps. Mechanical stimulation was performed for 15 mins, after which point, samples were placed back into culture within the SCI-Tive workstation until the following day.

***PCR Analysis of Chondrogenic Markers (Static vs Mechanical stimulation)***

One-step qRT-PCR was performed using the Superscript III Platinum SYBR green enzyme (Part #: 11736051; Invitrogen, Paisley, UK), according to manufacturers' instructions. Primers (F indicates Forward primer, R indicates Reverse primer, annealing temperature used is indicated for each primer pair) were purchased from Qiagen, UK (Aggrecan, ACAN, Part #: QT00001365; Collagen II, SLCO2A1, Part #: QT00095375; SOX-9, Part #: QT00001498 and GAPDH, Part #: QT01192646). All qRT-PCR reactions comprised a single cycle of 50 °C for 3 mins, a single cycle of 95 °C for 5 mins, followed by 40 cycles of 95 °C for 15 secs, annealing temp for 45 °C for 30 secs and 72 °C for 1 min; finally followed by a single cycle of 40 °C for 1 min using the thermo cycler machine. Raw $C_T$ values of interested genes were normalized to raw $C_T$ values of the housekeeping gene GAPDH to attain Δ $C_T$ values per gene. These values were then converted to ΔΔ $C_T$ values, which were the final values used in the graph.

***Evaluation of Cell Viability after Injection through Different Needle Gauges***



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Trypsinised hMSCs were resuspended homogenously into the polymer hydrogel solution (pre-photocuring) at a cell density of 100 000 cells / 50 µl polymer solution. hMSC/polymer solutions were injected using different hsurgical gauge size needles (19G, 22G, 27G and 30G; Terumo, UK) into a 6-well plate and incubated at 2% $O_2$ for 1hr and 24hrs after which cell viability was evaluated using trypan blue and live/dead staining solutions (FLUKA Analytical, Sigma-Aldrich), as used previously in our study [7].

***IVD Penetration Force Study***

Frozen Oxtail IVDs were thawed down to room temp prior to use. Various sized needles (22G, 25G, 27G and 30G) were fixed to a compressive testing machine (Model 5848; Instron Ltd, UK). This was achieved using a stainless steel adaptor (Figure 4A) that could receive a 2ml syringe barrel with reduced length and a tip for needle attachment (Terumo Ltd, UK). The bespoke adaptor attached to the mobile upper jaw of the testing machine.

The machine was set in compression mode with a 5N load cell installed and the mobile upper-jaw of the tester was programmed to travel at a rate of $1mm.s^{-1}$. The Ox tail was affixed to a polystyrene sheet using spare needles to prevent sample from moving during penetration (Figure 4B). For each experiment, the needle was pushed into the IVD by approximately 5mm. This penetration depth ensured the needle pushing past the annulus fibrosis and entered into the nucleus pulposus.



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## RESULTS AND DISCUSSION

### *Effects of 3D culture and cyclic compression*

In this study, further insights were examined in terms of specifying the power and impact of each of the stimulating parameters (hydrogel, hypoxia, chemical environment and mechanical stimulation) individually on the differentiation process of hMSCs towards an NP-cell like phenotype. PCR analysis revealed that 3D culture of hMSCs within the hydrogel under hypoxic conditions triggered the differentiation of hMSCs towards an NP-cell like phenotype (**Figure 1)**. This was evidently shown through elevated gene expression of SOX-9 (**Figure 1A**), aggrecan (**Figure 1B**) and collagen II (**Figure 1C**) at day 14 when compared to 2D culture, in hMSC media. When combining cell-gel constructs under hypoxia and in chondrogenic media, gene expression of SOX-9 (**Figure 1A**), Aggrecan (**Figure 1B**) and Collagen II (**Figure 1C**) were further elevated at day 14. However, the significant decrease in gene expression from day 7 to day 14, when cell hydrogel constructs were in hypoxia and chondrogenic media, may have been attributed to the complete transcriptional switch from a stem cell to a terminally differentiated NP cell-like phenotype and that translational expression of matrix proteins could take place after day 7 during in vitro culture.

We next integrated mechanical stimulation to the study and investigated the effects of 5% cyclic compression, 1Hz, 15 mins a day and up to day 14, on hMSCs encapsulated within hydrogels, under hypoxia, in hMSC or chondrogenic media. Immunocytochemistry was used to reveal the translational expression of key NP matrix proteins aggrecan and collagen II. Minimal differences in expression of Agg and Coll II were witnessed when culturing hMSCs (2D, on tissue culture plastic (TCP); or 3D, cells encapsulated within hydrogels) in hMSC media under hypoxia



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**(Figure 2A and 2C**). However, it was apparent that after the addition of chondrogenic media, further elevated levels of aggrecan and collagen II were witnessed when cultured under hypoxic conditions for 14 days (**Figure 2B and 2D)**. Interestingly, the greatest level of translational expression of both aggrecan and collagen II was witnessed in hydrogels cultured in hypoxia and chondrogenic media and exposed to mechanical stimulation up to day 14 **(Figure 2)**. This *in vitro* 3D culture with mechanical stimulation demonstrated the ability to support hMSC viability and differentiation towards an NP cell-like phenotype. However, further improvements to the model can be made in order to mimic the mechanical stimulation parameters close to the biomechanics of IVD by applying more long-term mechanical stimulation regime.



***Post-injection viability of cells***

Coupled together, with the ability of the hydrogels to provide protection to the encapsulated cells and mechanical support to the surrounding tissue *in vivo*, the ability to deliver cells in a minimally invasive procedure to the target site as well as the ability of the hydrogel to photocure *in situ* after delivery, encouraged the consideration in the delivery parameters for a device which would be used for local delivery of cell-laden hydrogel solution *in vivo*. To minimize the damage to the IVD from the needle penetration, needles with smaller diameter are ideal. However, narrower needle gauges could cause damage to the cells due to shear stresses imposed within the needle bore. Hence, the logical step in determining the most suitable needle gauge (**Figure 3A)** to deliver hMSC-laden hydrogels was to establish the effect of the inner diameter of the needle used on cell viability after delivery.



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Heng et al 2012 [9] have reported that passing hMSCs through a narrower catheter (26G nitinol needle lumen) at flow rates, which are normally encountered, resulted in no changes in hMSC multipotency or viability. This was an intramyocardial injection of hMSCs directly into heart muscle. In another study by Garvican et al, 2014 [10]; no changes in stem cell differentiation was observed when injected through 19G, 21G or 23G needles. However, there was a compromise in viability injecting through 21G and 23G needles. The general consensus therefore appears to be the preference by clinicians for smaller bore needles for hMSC delivery [11].

As the proposed route of delivery is to: 1) inject cell hydrogel solution *in situ* followed by, 2) cross-linking using a UV source, it was imperative to investigate various needle gauges (22G, 25G, 27G and 30G) and the impact on cell viability. Observations revealed that after injection, hMSCs retained good cell viability on TCP with the ability to fully recover their typical fibroblastic morphology in 24 hrs (**Figure 3B)**. Insignificant differences in viability were observed between 22G, 25G and 27G, although significant differences in cell viability were witnessed with 30G compared to all other sizes (**Figure 3B and C)**. This data lies in agreement with literature suggesting the use of 22G to 27G needles. The minimal cell death maybe attributed to the masking/protection of cells through the viscosity of the polymer from the impact of shear forces applied by these needles [12,13]. However, a limitation of this experiment includes the inability to control the flow rates precisely, which could contribute to the large standard deviation on average number of dead cells observed in Figure 3C. Future studies could include delivery using various flow rates controlled by a electrical motor, but we feel the manual injection would reflect the manner of



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stem cells delivery more close to the medical procedures deployed in a clinical setting [14,15].

After delivery, photo-crosslinking of cell loaded hydrogel solution would take place in situ where our previous study and others have confirmed insignificant compromise on viability using the photoinitiator (Irgacure 2959) and the UV parameters [7]. In addition, no secondary surgical intervention would be required after therapy, as the hydrogel is designed/tailored to breakdown with degradation products less than 20kDa (limit for renal filtration), allowing smooth excretion of the polymer-by-products through the metabolic pathway efficiently, preventing subsequent localised accumulation of hydrogel by-products [16].

***Quantification of tissue penetration force***

Needle penetration forces into IVD were investigated using an ex vivo tissue model. Explant IVD tissue from Ox tail (with similar dimensions and mechanical properties to human IVD) was penetrated using different needle gauges (**Figure 4A-C)**. Greater variation in penetration force was observed using larger needle gauge and is probably attributed to the larger outer diameter of the needle resulting in greater and controlled tearing of the tissue as it penetrates through the Annulus Fibrosus (AF) (**Figure 4D)**. The 22G needle was shown to require the greatest mean force to penetrate 2.17N (1.39%SD), the 30G needle was shown to have the lowest force to penetrate with the penetration point being the most consistent with a mean of 0.51 N (0.04% SD) (**Figure 4D)**. Smaller penetration forces were encountered using smaller needles, but it is also important to ensure that cell viability is not compromised through direct shear forces, although this does not appear to be the case here. Hence, the selection of delivery device parameters would be a negotiation between the internal diameter to



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ensure cell viability and the request of a small needle gauge to ensure negligible effect on IVD integrity [17,18]. It is anticipated that a suitable minimally invasive delivery device must be able to exert approximately a force of 2.5N, which is required to successfully penetrate through the AF of an IVD. The needle gauge of the device could be in the range of 19-30G, although using a smaller bore needle can minimise penetration forces, but also it will increase the pressure required on the back of a syringe, most likely when a viscous hydrogel solution is delivered.

***Delivery device deign***

Having taken all parameters and outcomes into consideration, the formulated CAD model of the chosen delivery design is displayed in **Figure 4D**. Major design components include: a consumable cell reservoir of hMSCs and hydrogel solution, battery-powered stepper motor with controlled injection and corresponding control and feedback components. This allows accurate and precise volumetric delivery via an electromechanical device facilitating intelligent control of the delivery force. The possibility of a battery device improves its ergonomic performance, as no wires would hinder the final positional control by the surgeon. Briefly, injection of hMSC loaded hydrogel solution is controlled through a rocker trigger activated with the users forefinger. However, further investigations would be required to perform proof of concept studies using the device with an *in vivo* pre-clinical model (bovine or ovine).

Preliminary assessment of the injectable parameters has provided insights into the design of an injection device capable of delivering a UV-curable hydrogel loaded with hMSCs that could be used to inject through the AF and into IVD. It is anticipated that the delivery method would be minimally invasive. This is attributed to the fact that the device would have a narrow enough needle to penetrate through the AF and



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into the NP. The cannula would then be changed to allow injection/delivery of the hMSC loaded polymer solution with the photo-initiator to the delivery site. Following delivery, a probe would then followed through the catheter to the target site allowing the exposure of UV light with optimized parameters to permit photocuring in situ. Once cured the properties of the hydrogel would help to restore mechanical function of IVD for the time being [2,19] until developmental state of hMSCs and cell/scaffold interactions are proper for effective ECM production, hence, contributing to the repair of NP tissue [5,20,21].

**Conclusion**

This study has demonstrated the successful use of a UV photocurable, synthetic, hydrogel in encapsulating hMSCs and differentiating them towards an NP cell-like phenotype when cultured in a combined environment of hypoxia, chemical stimulation and mechanical stimulation. In addition, we have considered the delivery route of the cell loaded hydrogel solution in situ, have demonstrated adequate cell viability and morphology after injection using 22G and 27G needles and quantified the force required for successful penetration through the AF.





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**Disclosure:** The authors declare no conflicts of interest.

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## FIGURE LEGENDS

**Figure 1.** Gene expression. Real time PCR demonstrating the expression of typical IVD markers; (A) SOX-9, (B) Aggrecan, (C) Collagen II in primary chondrocytes (OK cells) and hMSCs cultured in 2D and 3D (hydrogels) environments, in hMSC or chondrogenic media, under static or stimulated conditions in hypoxia up to 14 days. Annotations: Human Chondrocytes (OK), MSCs cultured in MSC expansion media (MSC MM), MSCs cultured in chondrogenic media (MSC CM), on tissue culture plastic (2D) and within hydrogels (3D).

**Figure 2.** IVD matrix protein expression. Immunocytochemistry demonstrating the expression of typical IVD matrix proteins; Collagen II expression in hMSCs cultured within hydrogels under static and stimulated conditions at day 7 and day 14 of culture in: (A) hMSC differentiation media and (B) in chondrogenic media. Aggrecan expression in hMSCs cultured within hydrogels under static and stimulated conditions at day 7 and day 14 of culture in : (C) hMSC differentiation medium and (D) chondrogenic medium.

**Figure 3**. Injection delivery route evaluation using different needle gauge sizes. (A) Defines the dimensions of the different sized gauge needles used to inject hMSCs into a well plate. (B) hMSC viability (live/dead staining) 24 hours after injection through various sized needle gauges. (C) Quantification of the percentage of hMSC dead cells (trypan blue) 1 hour after injection through various sized needle gauges.



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**Figure 4**. IVD penetration force in an *ex vivo* bovine model. (A) Bespoke adaptor assembled with the syringe component to connect the various sized needle gauges used in the study, (B) sectioned oxtail vertebrae, (C) *ex vivo* tissue being injected using a needle mounted on the materials tester, (D) box plot revealing IVD penetration forces required by the various needle gauge sizes used, (E) CAD design visualization of a potential injection device to deliver UV curable hydrogel (left) and a UV light source (right) for the treatment of IVD *in vivo*.





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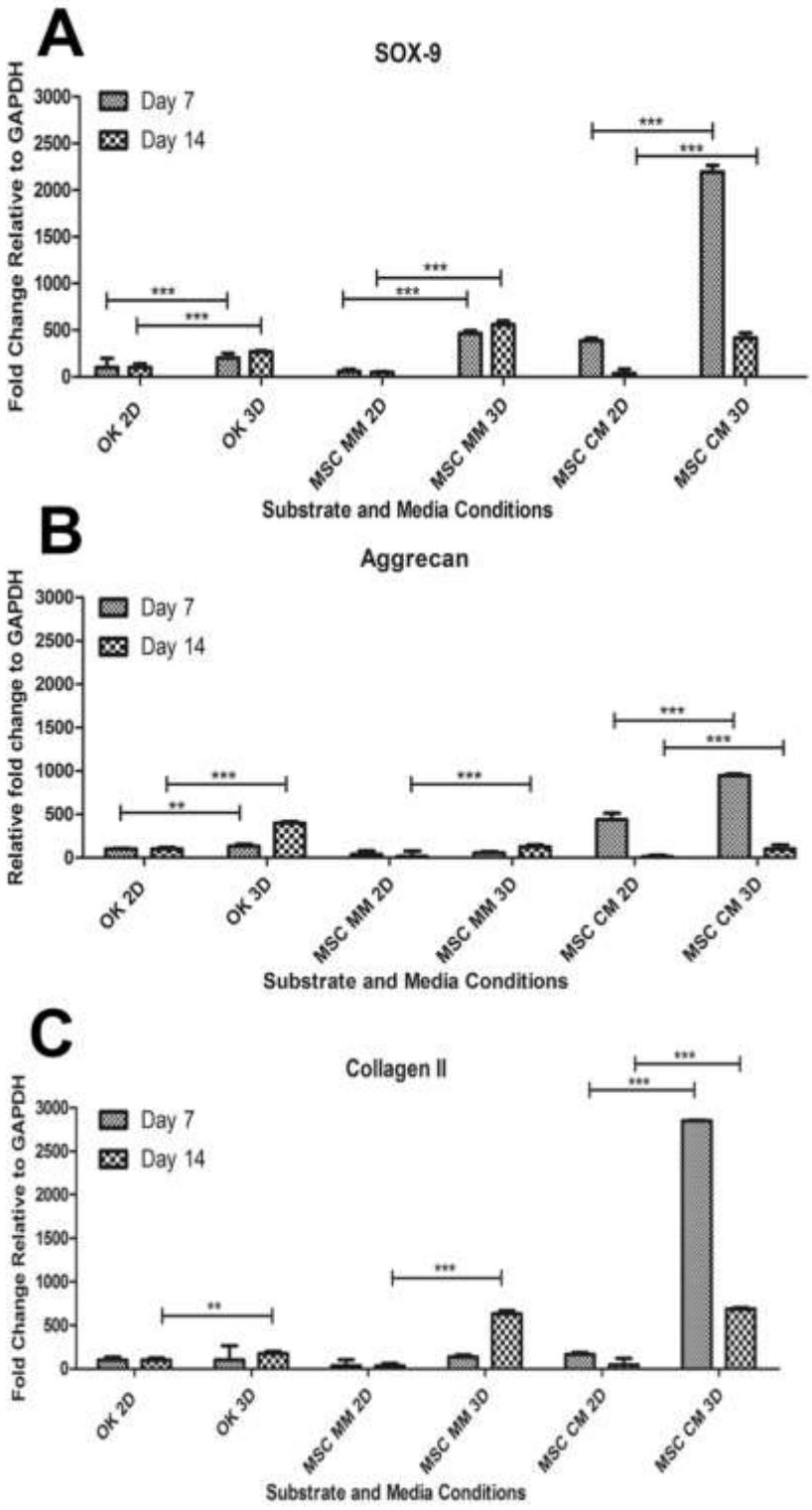


Figure 1



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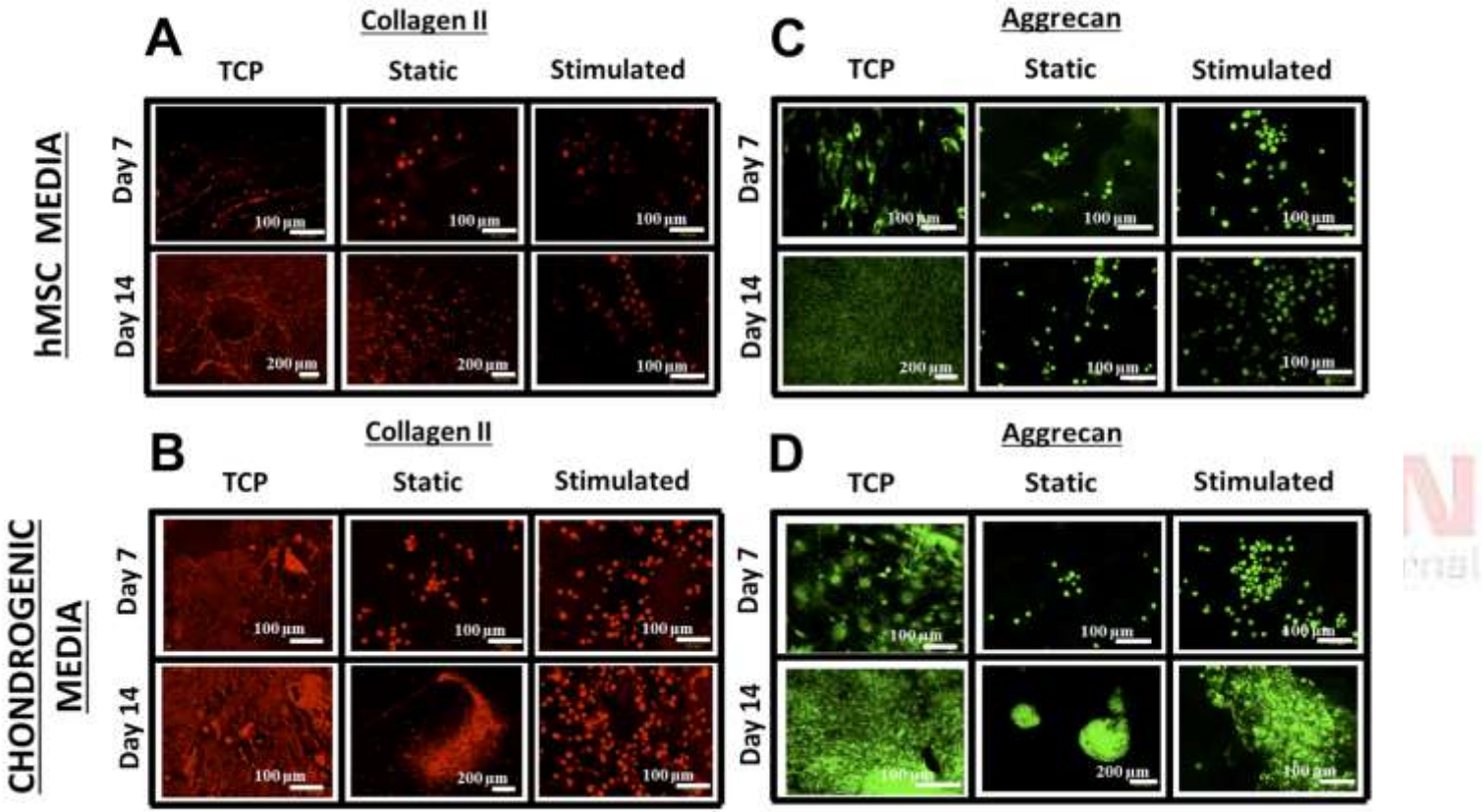
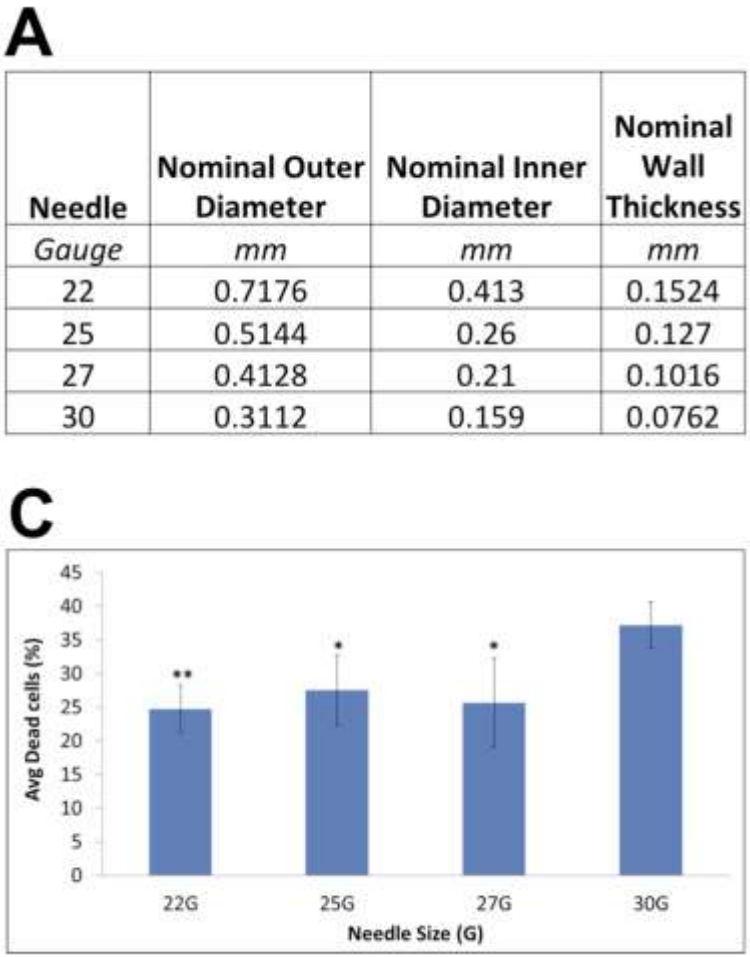



Figure 2

A

| Needle | Nominal Outer Diameter | Nominal Inner Diameter | Nominal Wall Thickness |
|---|---|---|---|
| *Gauge* | *mm* | *mm* | *mm* |
| 22 | 0.7176 | 0.413 | 0.1524 |
| 25 | 0.5144 | 0.26 | 0.127 |
| 27 | 0.4128 | 0.21 | 0.1016 |
| 30 | 0.3112 | 0.159 | 0.0762 |



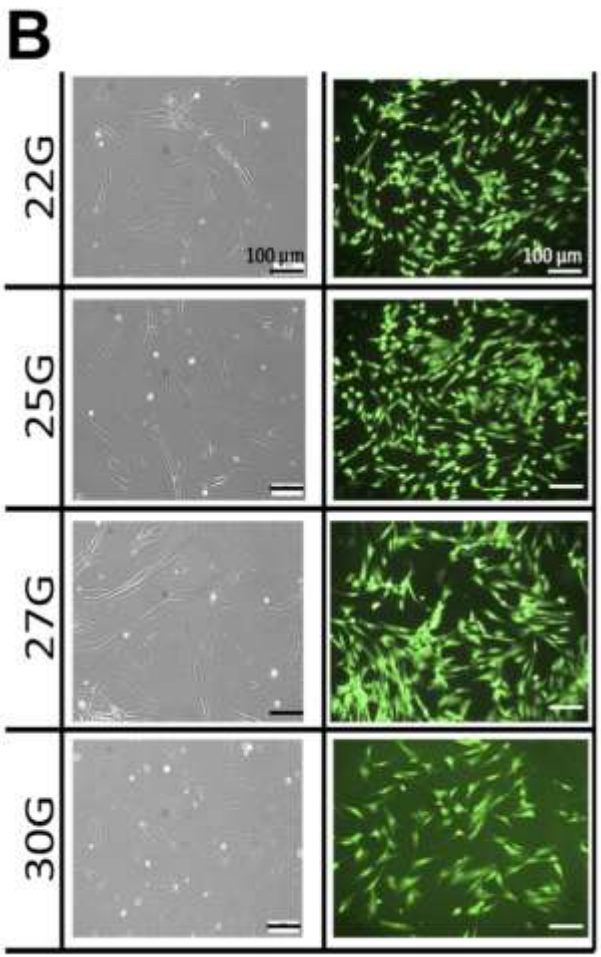




Figure 3

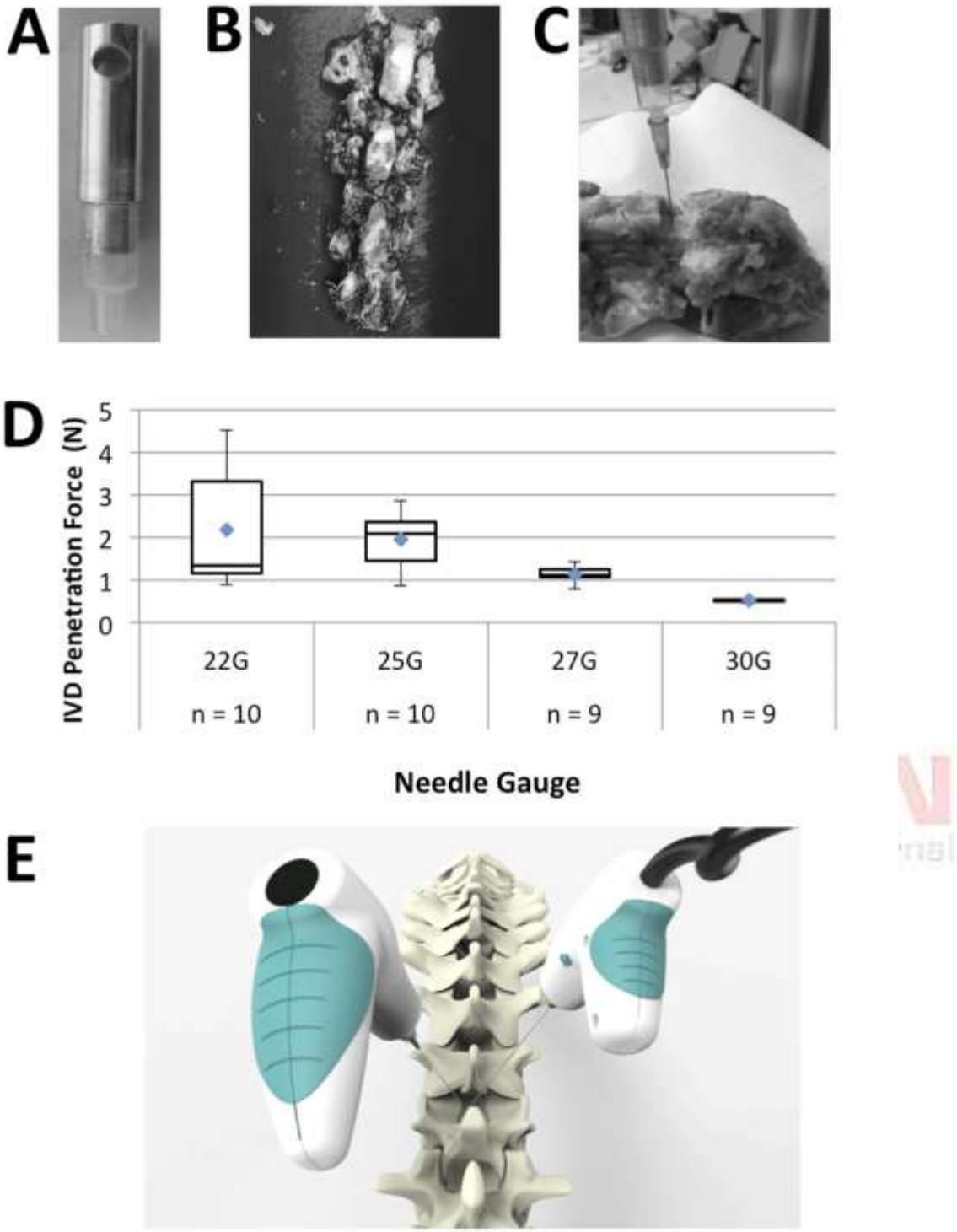



Figure 4



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